Remarks

The final Office Action mailed May 16, 2007 has been carefully reviewed and the foregoing amendment has been made in consequence thereof.

Claims 31, 33, 34, 36, and 43-51 are now pending in this application. Claims 1-30, 32, 35, 37-42 have been canceled. Claims 31-42 stand rejected. Claims 43-51 are newly added. Applicant submits that no new matter has been added.

Applicants would like to thank the Examiner for taking time to discuss the Office Action during a telephonic interview on August 1, 2007. During the interview, Applicant and the Examiner discussed the rejection of claims 31-42 due to a lack of a minimal promoter. Accordingly, Applicant has amended all claims in this application to incorporate a minimal promoter for the viable transcription of the transgene. During the interview, Applicant and the Examiner also discussed the rejection of Claims 38-40 as failing to comply with the written description requirement for describing regulatory DNA sequences specific to the heart, cartilage, and bone. To address these issues, Applicant has included references to examples of specific DNA regulatory sequences that are isolated from heterologous species, but which are highly conserved across phyla and can be used to effect cell specific expression of transgenes in transgenic fish regarding heart tissue, cartilage, and bone. These references are discussed in more detail below. In addition, Applicant has provided new claims and associated prior art references (also discussed below) for a number of other cell types and tissues, all of which were specifically listed in the specification as being "of interest" regarding "the cellular and/or tissue specific expression of an ablation-promoting moiety in transgenic zebrafish" (see paragraph [0070]).

The objection to Claims 33 and 34 is respectfully traversed. Applicants have amended Claims 33 and 34 to address the issues raised in the Office Action. Accordingly, Applicant respectfully requests that the objection to Claims 33 and 34 be withdrawn.

The rejection of Claims 38-40 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement is respectfully traversed.

Applicant submits that paragraph [0070] of the specification recites that "[r]egarding the cellular and/or tissue specific expression of an ablation-promoting [moiety] in transgenic zebrafish as disclosed herein; of interest are those cells, cell types or tissues that are common to humans and zebrafish. That is, those elements of the human system that are modeled in corresponding zebrafish systems. Such systems include, but are not limited to: (i) the nervous system - e.g., retina; (ii) the vascular system; (iii) the skeletal system; (iv) muscle; (v) the enteric system - e.g., liver. Of particular interest, is expression in those cells, cell types, or tissues relevant to modeling specific degenerative diseases in zebrafish. Also of interest, is expression in specific cells, cell types, or tissues whose degeneration is thought to be causative and/or otherwise linked to the etiology and/or symptoms of a given degenerative disease. For instance, the symptoms of Parkinson's disease are believed to be caused by the loss of dopamine, more specifically the loss of dopaminergic neurons. Therefore, regulatory DNA sequences of a gene which is active in dopaminergic neurons are utilized for specific expression of a transgene product in dopaminergic neurons. Particularly useful for targeting expression in discrete neuronal subpopulations, are genes required for the biosynthesis and/or transport of neurotransmitters. Accordingly, promoter elements of the dopamine transporter (DAT) are used to specifically express transgene products in dopaminergic neurons for the purpose of creating a zebrafish model of Parkinson's disease."

Further, the following prior art references support Claims 38-40 and newly added Claims 46-50. These references relate to examples of specific DNA regulatory sequences isolated from heterologous species, but which are highly conserved across phyla, that can be used to effect cell specific expression of transgenes in transgenic fish regarding heart, cartilage, bone, muscle, glial, pancreatic, liver, kidney, and vascular cells.

References for regulatory sequences driving transgene expression specifically in heart cells include the following:

1) Small EM, and Krieg PA.(2000). Expression of atrial natriuretic factor (ANF) during Xenopus cardiac development. Dev Genes Evol. 210:638-40. Department of Cell Biology and Anatomy, University of Arizona Health Science Center, P.O. Box 245044, 1501 N. Campbell Avenue, Tucson, AZ 85724, USA.

> We have isolated the Xenopus orthologue of the atrial natriuretic factor (ANF) gene. Characterization of embryonic expression indicates that the ANF gene is initially expressed throughout the developing myocardium at the late heart tube stage (about stage 32). This is in contrast to all previously characterized Xenopus cardiac differentiation markers that are first expressed in the cardiogenic plate at approximately stage 27. ANF expression becomes restricted exclusively to the atrium at about stage 47, long after the commencement of beating and the original formation of the atrial and ventricular compartments, but shortly after septation of the single atrium into two distinct atria.

2) Lien CL, Wu C, Mercer B, Webb R, Richardson JA, Olson EN.(1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. Development. 126:75-84. Departments of Molecular Biology and Oncology and Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235-9148, USA.

> The homeobox gene Nkx2-5 is the **earliest known marker of the cardiac lineage** in vertebrate embryos. Nkx2-5 expression is first detected in mesodermal cells specified to form heart at embryonic day 7.5 in the mouse and **expression is maintained throughout the developing and adult heart**. In addition to the heart, Nkx2-5 is transiently expressed in the developing pharynx, thyroid and stomach. To investigate the mechanisms that initiate cardiac transcription during embryogenesis, we analyzed the Nkx2-5 upstream region for regulatory elements sufficient to direct expression of a lacZ transgene in the developing heart of transgenic mice. We describe a **cardiac enhancer**, located about 9 kilobases upstream of the Nkx2-5 gene, that fully recapitulates the expression pattern of the endogenous gene in cardiogenic precursor cells from the onset of cardiac lineage specification and throughout the linear and looping heart tube. Thereafter, as the atrial and ventricular chambers become demarcated, enhancer activity becomes restricted to the developing right ventricle. **Transcription of Nkx2-5 in pharynx, thyroid and stomach is controlled by regulatory elements separable from the cardiac enhancer**. This distal cardiac enhancer contains a high-affinity binding site for the cardiac-restricted zinc finger transcription factor GATA4 that is essential for transcriptional activity. These results reveal a novel GATA-dependent mechanism for activation of Nkx2-5 transcription in the developing heart and indicate that regulation of Nkx2-5 is controlled in a modular manner, with multiple regulatory regions responding to distinct transcriptional networks in different compartments of the developing heart. (Emphasis added)

3) RJ Schwartz and EN Olson (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. Development, 126:4187-4192. Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

> Heart formation in Drosophila is dependent on the homeobox gene tinman. The homeobox gene Nkx2-5 is closely related to tinman and is the earliest known marker for cardiogenesis in vertebrate embryos. Recent studies of cis-regulatory elements required for Nkx2-5 expression in the developing mouse heart have revealed an extraordinary array of independent cardiac enhancers, and associated negative regulatory elements, that direct transcription in distinct regions of the embryonic heart. These studies demonstrate the **modularity in cardiac transcription**, in which **different regulatory elements respond to distinct sets of transcription factors to control gene expression in different compartments of the developing heart**. We consider the potential mechanisms underlying such transcriptional complexity, its possible significance for cardiac function, and the implications for evolution of the multichambered heart. (Emphasis added)

4) Durocher D, and Nemer M. (1998). Combinatorial interactions regulating cardiac transcription. Dev Genet., 22:250-62. Laboratoire de developpement et differenciation cardiaques, Institut de recherches cliniques de Montreal, Quebec, Canada.

> In vertebrates, heart development is a multistep process that starts with formation and patterning of the primitive heart tube and is followed by complex morphological events to give rise to the mature four-chambered heart. These various stages are characterized by distinct patterns of gene expression. Although chamber specificity and developmental regulation can be demonstrated in transgenic mice using short promoter fragments, the mechanism underlying spatial and temporal specificity within the heart remains largely unclear. Combinatorial interaction between a limited number of cardiac-specific and ubiquitous transcription factors may account for the diverse genetic inputs required to generate the complex transcriptional patterns that characterize the developing myocardium. We have used the cardiac atrial natriuretic peptide (ANP) promoter to test this hypothesis. The **ANP gene is transcribed in a spatial- and temporal-specific manner in the heart, and a 500 bp promoter fragment is sufficient to recapitulate both chamber and developmental specificity**. This promoter is composed of three modules, a "basal" cardiac promoter that is essential for transcription in embryonic and postnatal atrial and ventricular myocytes and two other independent modules that behave as chamber-specific enhancers. The basal cardiac promoter is the target of two cardiac-specific transcription factors, the zinc finger GATA-4 protein and the Nkx2-5 homeodomain, which bind to

> contiguous elements within this region. At low concentrations--a situation that likely occurs during the very first stages of cardiac cell fate determination--the two proteins synergistically activate transcription from the ANP promoter. This functional synergy requires physical interaction between the GATA-4 protein and an extended C-terminal homeodomain on Nkx2-5. This interaction, which unmasks an activation domain present just N-terminal of the homeodomain, is specific for GATA-4 and-5, but is not observed with the other cardiac GATA factor, GATA-6. Optimal synergy requires binding of both proteins to their cognate sites, although modest synergy also could be observed on heterologous promoters containing only multimerized Nkx binding sites, suggesting that Nkx2-5 is able to recruit GATA-4 into a transcriptionally active complex. The GATA/Nkx interaction, which appears to have been evolutionary conserved in nematode, fly, and mammals, provides a paradigm for analyzing transcription factor interaction during organogenesis. The data are also discussed in the context of our present knowledge of the roles of GATA and NK2 proteins in cardiac development. (Emphasis added)

5) Qian Q, Kuo L, Yu YT, and Rottman JN. (1999). A concise promoter region of the heart fatty acid-binding protein gene dictates tissue-appropriate expression. Circ Res. 84:276-89. Departments of Internal Medicine (Cardiology), Vanderbilt University School of Medicine, Nashville, TN, USA.

> The heart fatty acid-binding protein (HFABP) is a member of a family of binding proteins with distinct tissue distributions and diverse roles in fatty acid metabolism, trafficking, and signaling. Other members of this family have been shown to possess concise promoter regions that direct appropriate tissue-specific expression. The basis for the **specific expression of the HFABP** has not been previously evaluated, and the mechanisms governing expression of metabolic genes **in the heart** are not completely understood. We used transient and permanent transfections in ventricular myocytes, skeletal myocytes, and nonmyocytic cells to map regulatory elements in the HFABP promoter, and audited results in **transgenic mice**. **Appropriate tissue-specific expression** in cell culture and in transgenic mice was dictated by **1.2 kb of the 5'-flanking sequence of FABP3, the HFABP gene**. Comparison of orthologous murine and human genomic sequences demonstrated multiple regions of near-identity within this promoter region, including a CArG-like element close to the TATA box. Binding and transactivation studies demonstrated that this element can function as an atypical myocyte enhancer-binding factor 2 site. Interactions with adjacent sites are likely to be necessary for fully appropriate, tissue-specific, developmental and metabolic regulation. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in cartilage cells include the following:

1) Doege K, Hall LB, McKinnon W, Chen L, Stephens DT, and Garrison K. (2002). A remote upstream element regulates tissue-specific expression of the rat aggrecan gene. J Biol Chem. 277:13989-97. Epub 2002 Feb 7. Department of Biochemistry, College of Medicine, University of South Florida, Tampa, Florida 33612, USA.

> The regulation of chondrogenesis and of the genes expressed as markers of chondrocyte differentiation is poorly understood. The hyaluronan-binding proteoglycan aggrecan is an essential and specific component of cartilage, but the aggrecan proximal promoter is expressed in an unregulated fashion in vitro. DNA comprising the rat aggrecan gene (83 kb including the 30-kb first intron) was surveyed for active elements, which would impart selective expression to the aggrecan promoter in transfection assays in vitro. A 4.7-kb DNA fragment (P3) with cell-specific enhancer activity was discovered approximately 12 kb upstream of the transcription start site; this active DNA fragment is position- and orientation-independent, and strongly stimulates aggrecan promoter expression in chondrocytes, while weakly suppressing transcription in fibroblasts. Most of this activity has been localized to P3-7, a 2.3-kb internal fragment of P3. Another enhancer element (A23), which is not tissue-specific, was discovered about 70 kb downstream of the transcription start site. Several lines of transgenic mice were created using combinations of these DNA elements to drive the lacZ reporter gene. Neither a short (900 bp) nor a long (3.7 kb) promoter alone showed detectable expression in 14.5-day embryos, whereas placing the **P3 tissue-specific enhancer together with P0** gave strong expression restricted to embryonic **cartilage** of **transgenic mice**. The A23 downstream enhancer in conjunction with P0 did not confer expression. This is the first report of a gene control region which confers authentic tissue-specific regulation of aggrecan in vitro or in vivo and should greatly facilitate understanding the coordinate regulation of chondrocytic genes. (Emphasis added)

2) Pirok EW 3rd, Henry J, and Schwartz NB. (2001) cis elements that control the expression of chick aggrecan. J Biol Chem. 276:16894-903. Department of Pediatrics, University of Chicago, Chicago, Illinois 60637, USA.

> **Aggrecan** is a large chondroitin sulfate proteoglycan whose expression is both cell-specific and developmentally regulated. Cloning and sequencing of the 1.8-kilobase genomic 5'-flanking sequence of the chick aggrecan gene revealed the presence of potential tissue-specific control elements including a consensus sequence found in the **cartilage-associated**

> **silencers**, CSIIS1 and CSIIS2, that were first characterized in the type II collagen promoter sequences, as well as numerous other cis elements. Transient transfections of chick sternal chondrocytes and fibroblasts with reporter plasmids bearing progressively deleted portions of the chick aggrecan promoter and enhancer region demonstrated **cell type-specific promoter activity** and identified a 420-base pair region in the genomic 5-flanking region responsible for negative regulation of the aggrecan gene. In this report, three complementary methods, DNase I footprinting assays, transient transfections, and electrophoretic mobility shift assays (EMSA), provided an integral approach to better understand the regulation of the aggrecan gene. DNase I footprinting revealed that six regions of this genomic sequence bind to nuclear proteins in a tissue-specific manner. Transient transfection of reporter constructs bearing ablations of these protected sequences showed that four of the six protected sequences, which contain the sequence TCCTCC or TCCCCT, had repressor activities in transfected chick chondrocytes. Cross-competition EMSA using nuclear protein extracted from chondrocytes or fibroblasts explored the contributions of the different sequence elements in formation of DNA-protein complexes specific to cell type. This is the first parallel examination of the EMSA patterns for six functionally defined cis elements with highly similar sequences, using protein from primary cultured cells. (Emphasis added)

3) Lefebvre V, Zhou G, Mukhopadhyay K, Smith CN, Zhang Z, Eberspaecher H, Zhou X, Sinha S, Maity SN, and de Crombrugghe B. (1996). An 18-base-pair sequence in the mouse proalpha1(II) collagen gene is sufficient for expression in cartilage and binds nuclear proteins that are selectively expressed in chondrocytes. Mol Cell Biol.16:4512-23. Department of Molecular Genetics, The University of Texas M.D. Anderson Cancer Center, Houston 77030, USA.

> The molecular mechanisms by which mesenchymal cells differentiate into chondrocytes are still poorly understood. We have used the gene for a chondrocyte marker, the proalpha1(II) collagen gene (Col2a1), as a model to delineate a minimal sequence needed for chondrocyte expression and identify chondrocyte-specific proteins binding to this sequence. We previously localized a cartilage-specific enhancer to 156 bp of the mouse Col2a1 intron 1. We show here that four copies of a 48-bp subsegment strongly increased promoter activity in transiently transfected rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not in 10T1/2 fibroblasts. They also directed cartilage specificity in transgenic mouse embryos. These 48 bp include two 11-bp inverted repeats with only one mismatch. Tandem copies of an 18-bp element containing the 3' repeat strongly enhanced promoter activity in RCS cells and chondrocytes but not in fibroblasts. Transgenic

mice harboring 12 copies of this 18-mer expressed luciferase in ribs and vertebrae and in isolated chondrocytes but not in noncartilaginous tissues except skin and brain. In gel retardation assays, an RCS cell-specific protein and another closely related protein expressed only in RCS cells and primary chondrocytes bound to a 10-bp sequence within the 18-mer. Mutations in these 10 bp abolished activity of the multimerized 18-bp enhancer, and deletion of these 10 bp abolished enhancer activity of 465- and 231-bp intron 1 segments. This sequence contains a low-affinity binding site for POU domain proteins, and competition experiments with a high-affinity POU domain binding site strongly suggested that the chondrocyte proteins belong to this family. Together, our results indicate that an 18-bp sequence in Col2a1 intron 1 controls chondrocyte expression and suggest that RCS cells and chondrocytes contain specific POU domain proteins involved in enhancer activity.

4) Zhou G, Garofalo S, Mukhopadhyay K, Lefebvre V, Smith CN, Eberspaecher H, de Crombrugghe B. (1995). A 182 bp fragment of the mouse pro alpha 1(II) collagen gene is sufficient to direct chondrocyte expression in transgenic mice. J Cell Sci. 108:3677-84. Department of Molecular Genetics, University of Texas, M. D. Anderson Cancer Center, Houston 77030, USA.

Type II collagen is a major chondrocyte-specific component of the cartilage extracellular matrix and it represents a typical differentiation marker of mature chondrocytes. In order to delineate cis-acting elements of the mouse pro alpha 1(II) collagen gene that control chondrocyte-specific expression in intact mouse embryos, we generated transgenic mice harboring chimeric constructions in which varying lengths of the promoter and intron 1 sequences were linked to a beta-galactosidase reporter gene. A construction containing **a 3,000 bp promoter and a 3,020 bp intron 1 fragment directed high levels of beta-galactosidase expression specifically to chondrocytes**. Expression of the transgene coincided with the temporal expression of the endogenous gene at all stages of embryonic development. **Successive deletions of intron 1 delineated a 182 bp fragment which targeted beta-galactosidase expression to chondrocytes with the same specificity as the larger intron 1 fragment**. Transgenic mice harboring a 309 bp Col2a1 promoter lacking intron 1 tester sequences showed no beta-galactosidase expression in chondrocytes. Reduction of the 182 bp fragment to a 73 bp subfragment surrounding a decamer sequence previously reported to be involved in chondrocyte specificity, resulted in loss of transgene expression in chondrocytes. When the Col2a1 promoter was replaced with a minimal beta-globin promoter, the 182 bp intron 1 sequence was still able to target expression of the transgene to chondrocytes. We conclude that a 182 bp

> intron 1 DNA segment of the mouse Col2a1 gene contains the necessary information to confer high-level, temporally correct, chondrocyte expression on a reporter gene in intact mouse embryos and that Col2a1 promoter sequences are dispensable for chondrocyte expression. (Emphasis added)

5) Gaiser KG, Maddox BK, Bann JG, Boswell BA, Keene DR, Garofalo S, and Horton WA. (2002). Y-position collagen II mutation disrupts cartilage formation and skeletal development in a transgenic mouse model of spondyloepiphyseal dysplasia. J Bone Miner Res. 17:39-47. Research Center, Shriners Hospital for Children, Portland, Oregon 97201, USA.

> Mice were generated by pronuclear injection of a type II collagen transgene harboring an Arg789Cys (R789C) mutation that has been found in patients with spondyloepiphyseal dysplasia (SED). **Expression was directed to cartilage by the murine Col2a1 promoter** to examine the consequences of mutations involving the Y-position of the collagen helix Gly-X-Y triplet on skeletogenesis. The transgenic mice had very short limbs, short trunk, short snout, and cleft palate; they died at birth. Their growth plates were disorganized and collagen fibrils were sparse in cartilage matrix. When the transgene was expressed in RCS cells, there was no evidence that R789C-bearing collagen chains were incorporated into stable collagen molecules. Molecular modeling of the mutation raised the possibility that it destabilizes the collagen triple helix. Together our results suggest that Y-position mutations, such as R789C, can act in a dominant negative manner to destabilize collagen molecules during assembly, reducing their availability to form fibrils, the deficiency of which profoundly disturbs the template functions of cartilage during skeletogenesis. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in bone cells include the following:

1) Dacic S, Kalajzic I, Visnjic D, Lichtler AC, and Rowe DW. (2001). Col1a1-driven transgenic markers of osteoblast lineage progression. J Bone Miner Res. 2001 16:1228-36. Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington 06030, USA.

> The modular organization of the **type I collagen promoter** allows creation of promoter-reporter constructs with preferential activity in different type I collagen-producing tissues that might be useful to mark cells at different stages of osteoblastic differentiation. Primary marrow stromal cell (MSC) and mouse calvarial osteoblast (mCOB) cultures were

established from transgenic mice harboring different Col1a1 promoter fragments driving chloramphenicol acetyltransferase (CAT). In these models, Col1a1 messenger RNA (mRNA) and alkaline phosphatase (ALP) are the first markers of differentiation appearing soon after the colonies develop. Bone sialoprotein (BSP) is detected 2-3 days later, followed by osteocalcin (OC) expression and nodule mineralization. A 3.6 Col1a1 fragment (ColCAT3.6) initiated activity concomitant with ALP staining and type I collagen mRNA expression. In contrast, a 2.3 Col1a1 fragment (ColCAT2.3) became active coincident with BSP expression. The pattern of transgene expression assessed by immunostaining was distinctly different. ColCAT3.6 was expressed within and at the periphery of developing nodules whereas the ColCAT2.3 expression was restricted to the differentiated nodules. The feasibility of using green fluorescent protein (GFP) as a marker of osteoblast differentiation was evaluated in ROS17/2.8 cells. A 2.3-kilobase (kb) Col1a1 promoter driving GFP (pOB4Col2.3GLP) was stably transfected into the cell line and positive clones were selected. Subcultures lost and then regained GFP expression that was localized in small clusters of cells throughout the culture. This suggests that expression from the 2.3-kb Col1A1 fragment is determined by the state of differentiation of the ROS17/2.8 cells. Col1a1 transgenes should be useful in appreciating the heterogeneity of a primary or immortalized culture undergoing osteoblastic differentiation. (Emphasis added)

2) Kalajzic I, Kalajzic Z, Kaliterna M, Gronowicz G, Clark SH, Lichtler AC, and Rowe D. (2002). Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J Bone Miner Res. 2002 Jan;17(1):15-25. Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington 06030, USA.

Green fluorescent protein (GFP)-expressing **transgenic mice** were produced containing a 3.6-kilobase (kb; pOBCol3.6GFPtpz) and **a 2.3-kb (pOBCol2.3GFPemd) rat type I collagen (Col1a1) promoter** fragment. The 3.6-kb promoter directed strong expression of GFP messenger RNA (mRNA) to bone and isolated tail tendon and lower expression in nonosseous tissues. The **2.3-kb promoter expressed the GFP mRNA in the bone and tail tendon with no detectable mRNA elsewhere**. The pattern of fluorescence was evaluated in differentiating calvarial cell (mouse calvarial osteoblast cell [mCOB]) and in marrow stromal cell (MSC) cultures derived from the transgenic mice. The pOBCol3.6GFPtpz-positive cells first appeared in spindle-shaped cells before nodule formation and continued to show a strong signal in cells associated with bone nodules. pOBCol2.3GFPemd fluorescence first appeared in nodules undergoing mineralization. Histological analysis

> showed weaker pOBCol3.6GFPtpz-positive fibroblastic cells in the periosteal layer and strongly positive osteoblastic cells lining endosteal and trabecular surfaces. In contrast, a pOBCol2.3GFPemd signal was limited to osteoblasts and osteocytes without detectable signal in periosteal fibroblasts. These findings suggest that Col1a1GFP transgenes are marking different subpopulations of cells during differentiation of skeletal osteoprogenitors. With the use of other promoters and color isomers of GFP, it should be possible to develop experimental protocols that can reflect the heterogeneity of cell differentiation in intact bone. In primary culture, this approach will afford isolation of subpopulations of these cells for molecular and cellular analysis. (Emphasis added)

3) Schinke T, and Karsenty G. (1999). Characterization of Osf1, an osteoblast-specific transcription factor binding to a critical cis-acting element in the mouse Osteocalcin promoters. J Biol Chem. 274:30182-9. Department of Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030, USA.

> To elucidate the mechanisms of osteoblast-specific gene expression we are studying the regulation of **osteocalcin, the most osteoblast-specific gene**. Previous studies of OG2, one of the two mouse osteocalcin genes, identified **two osteoblast-specific cis-acting elements**, OSE1 and **OSE2**, the latter being the **binding site of Cbfa1, the only osteoblast-specific transcription factor known to date**. Here we show that OSE1 is a cis-acting element as important as OSE2 for the osteoblast-specific expression of OG2 in cell culture and **transgenic mice**. We also show that OSE1 is present in the promoter of several osteoblast-specific genes including Cbfa1 itself. These biological features demonstrate the importance of OSE1 and led us to further characterize this site and the factor binding to it, provisionally termed Osf1. We first defined the core OSE1 sequence, 5'-TTACATCA-3', which is necessary and sufficient for Osf1 binding to DNA. This sequence has no strong homology to any known transcription factor-binding sites. As a first step in identifying Osf1, we performed an analytical purification of this protein using nuclear extracts from two different osteoblastic cell lines. We purified Osf1 to homogeneity through a five-step procedure including a renaturation experiment and found that its apparent molecular mass is 40 kDa. In conclusion, this study indicates the existence of multiple osteoblast-specific cis-acting elements of equal importance in controlling OG2 promoter activity and provides the first biochemical characterization of Osf1, a novel osteoblast-specific transcription factor. (Emphasis added)

4) Frendo JL, Xiao G, Fuchs S, Franceschi RT, Karsenty G, and Ducy P. (1998). Functional hierarchy between two OSE2 elements in the control of osteocalcin gene

expression in vivo. J Biol Chem. 273:30509-16. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA.

> **Osteocalcin gene** expression is initiated perinatally and is **restricted to mature osteoblasts** and odontoblasts. Because their pattern of expression is highly restricted, the osteocalcin genes are excellent tools to study osteoblast-specific gene expression. To define the mechanisms of osteocalcin cell-specific gene expression in vivo, we generated **transgenic mice** harboring deletion mutants of the promoter region of OG2, one of the mouse osteocalcin genes. We show here that **only 647 base pairs of this promoter are sufficient to confer cell-specific and time-specific expression to a reporter gene in vivo**. This promoter fragment contains two copies of OSE2. This osteoblast-specific cis-acting element binds Osf2, a recently characterized osteoblast-specific transcription factor (Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Cell 89, 747-754). We also demonstrate that the proximal OSE2 element is critical to confer an osteoblast-specific, developmentally regulated pattern of expression to a reporter gene. The other OSE2 element, located more upstream and presenting a lower affinity for Osf2, affects only weakly OG2 promoter activity. These data demonstrate the crucial role of Osf2 in controlling osteocalcin gene expression. Since osteocalcin synthesis is a hallmark of the differentiated osteoblast phenotype, these results suggest that, beyond its developmental function, Osf2 is also required for the maintenance of the osteoblast phenotype postnatally. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in muscle cells include the following:

1) Z Li, P Marchand, J Humbert, C Babinet and D Paulin (1993). Desmin sequence elements regulating skeletal muscle-specific expression in transgenic mice. Development, Vol 117, Issue 3 947-959. Biologie Moleculaire de la Differenciation, Universite Paris 7, France.

> During the development of the mouse embryo, desmin is one of the first muscle proteins detected in both the heart and the somites. The expression of the desmin gene differs from most other muscle genes, since it is initiated in replicating myoblasts and accumulates as the muscle differentiates. We have characterized a muscle-specific enhancer which directs the expression of desmin in vitro in the myoblasts and myotubes of C2 cells but not in non-myogenic cells. We report here on the generation and characterization of transgenic mice bearing a transgene in which the 1 kb DNA 5' regulatory sequence of the desmin gene is linked to a reporter gene coding for Escherichia

> coli beta-galactosidase (Des1-nlacZ). The enhancer activity of the desmin promoter is very strong and the reporter gene expression is easily detected in tissue sections. We have demonstrated that the **regulatory elements present in the transgene Des1-nlacZ are sufficient to direct muscle-specific and developmentally regulated expression of nlacZ in skeletal muscles**. Endogenous desmin expression and transgene activity were found to be correlated during the development of skeletal muscles. The transgene was expressed in the committed mononucleate myoblasts as well as in the myotubes. In addition, we have shown that the desmin-derived sequences direct a highly selective expression of nlacZ in cells that leave the somites and invade the limb bud, indicating that the cells that migrate from the somites are already predetermined for myogenesis. In contrast, smooth and cardiac muscle cells were beta-galactosidase negative both during embryonic and foetal development. Interestingly, the transgene was found to be expressed in the conduction system of the heart, which exhibits many features characteristic of skeletal muscles. (Emphasis added)

2) M Salminen, P Maire, J P Concordet, C Moch, A Porteu, A Kahn and D Daegelen (1994). Fast-muscle-specific expression of human aldolase A transgenes. Mol Cell Biol. 1994 October; 14(10): 6797-6808. Institut Cochin de Génétique Moléculaire, Université René Descartes, Paris, France.

> The expression of the human aldolase A gene is controlled by three alternative promoters. In transgenic mice, pN and pH are active in all tissues whereas pM is activated specifically in adult muscles composed mainly of fast, glycolytic fibers. To detect potential regulatory regions involved in the fast-muscle-specific activation of pM, we analyzed DNase I hypersensitivity in a 4.3-kbp fragment from the 5' end of the human aldolase A gene. Five hypersensitive sites were located near the transcription initiation site of each promoter in those transgenic-mouse tissues in which the corresponding promoter was active. Only one muscle-specific hypersensitive site was detected, mapping near pM. To functionally delimit the elements required for **muscle-specific activity of pM**, we performed a deletion analysis of the aldolase A 5' region in transgenic mice. Our results show that a **280-bp fragment containing 235 bp of pM proximal upstream sequences together with the noncoding M exon is sufficient for tissue-specific expression of pM**. When a putative MEF-2-binding site residing in this proximal pM region is mutated, pM is still active and no change in its tissue specificity is detected. Furthermore, we observed a modulation of pM activity by elements lying further upstream and downstream from pM. Interestingly, **pM was expressed in a tissue-specific way in all transgenic mice in which the 280-bp region was present**

> (32 lines and six founder animals). This observation led us to suggest that the proximal pM region contains elements that are able to override to some extent the effects of the surrounding chromatin. (Emphasis added)

3) P L Hallauer, K E Hastings, and A C Peterson (1988). Fast skeletal muscle-specific expression of a quail troponin I gene in transgenic mice. Mol Cell Biol. 1988 December; 8(12): 5072–5079. Ludwig Institute for Cancer Research, Montreal, Quebec, Canada.

> We have produced seven lines of **transgenic mice carrying the quail gene encoding the fast skeletal muscle-specific isoform of troponin I (TnIf)**. The quail DNA included the entire TnIf gene, 530 base pairs of 5'-flanking DNA, and 1.5 kilobase pairs of 3'-flanking DNA. In all seven transgenic lines, normally initiated and processed quail TnIf mRNA was expressed in skeletal muscle, where it accumulated to levels comparable to that in quail muscle. Moreover, in the three lines tested, quail TnIf mRNA levels were many fold higher in a fast skeletal muscle (gastrocnemius) than in a slow skeletal muscle (soleus). We conclude that the **cellular mechanisms directing muscle fiber type-specific TnIf gene expression are mediated by cis-regulatory elements present on the introduced quail DNA fragment** and that they control TnIf expression by affecting the accumulation of TnIf mRNA. **These elements have been functionally conserved since the evolutionary divergence of birds and mammals**, despite the major physiological and morphological differences existing between avian (tonic) and mammalian (twitch) slow muscles. In lines of transgenic mice carrying multiple tandemly repeated copies of the transgene, an aberrant quail TnIf transcript (differing from normal TnIf mRNA upstream of exon 2) also accumulated in certain tissues, particularly lung, brain, spleen, and heart tissues. However, this aberrant transcript was not detected in a transgenic line which carries only a single copy of the quail gene. (Emphasis added)

4) Cort S. Madsen, Christopher P. Regan, Jill E. Hungerford, Sheryl L. White, Ichiro Manabe, , Gary K. Owens (1998). Smooth Muscle–Specific Expression of the Smooth Muscle Myosin Heavy Chain Gene in Transgenic Mice Requires 5'-Flanking and First Intronic DNA Sequence. Circulation Research. 1998;82:908-917.

> The smooth muscle myosin heavy chain (SM-MHC) gene encodes a major contractile protein whose expression exclusively marks the **smooth muscle cell (SMC) lineage**. To better understand smooth muscle differentiation at the transcriptional level, we have initiated studies to identify those DNA sequences critical for expression of the SM-MHC gene. Here we **report the identification of an SM-MHC promoter-**

> ***intronic DNA fragment that directs smooth muscle–specific expression in transgenic mice.*** Transgenic mice harboring an SM-MHC-lacZ reporter construct containing {approx}16 kb of the SM-MHC genomic region from -4.2 to +11.6 kb (within the first intron) expressed the lacZ transgene in all smooth muscle tissue types. The inclusion of the intronic sequence was required for transgene expression, since 4.2 kb of the 5'-flanking region alone was not sufficient for expression. In the adult mouse, transgene expression was observed in both arterial and venous smooth muscle, in airway smooth muscle of the trachea and bronchi, and in the smooth muscle layers of all abdominal organs, including the stomach, intestine, ureters, and bladder. During development, transgene expression was first detected in airway SMCs at embryonic day 12.5 and in vascular and visceral SMC tissues by embryonic day 14.5. Of interest, expression of the SM-MHC transgene was markedly reduced or absent in some SMC tissues, including the pulmonary circulation. Moreover, the transgene exhibited a heterogeneous pattern between individual SMCs within a given tissue, suggesting the possibility of the existence of different SM-MHC gene regulatory programs between SMC subpopulations and/or of episodic rather than continuous expression of the SM-MHC gene. To our knowledge, results of these studies are the first to **identify a promoter region that confers complete SMC specificity in vivo**, thus providing a system with which to define SMC-specific transcriptional regulatory mechanisms and to design vectors for SMC-specific gene targeting. (Emphasis added)

5) Yanfei Xu, Jiangyan He, Ho Lian Tian, Chiew Hua Chan, Ji Liao, Tie Yan, Toong Jin Lam, Zhiyuan Gong (1999). Fast Skeletal Muscle-Specific Expression of a Zebrafish Myosin Light Chain 2 Gene and Characterization of Its Promoter by Direct Injection into Skeletal Muscle. DNA and Cell Biology. 1999, 18(1): 85-95.

> A zebrafish myosin light chain 2 cDNA clone was isolated and characterized. Sequence analysis of the clone revealed a high homology with the mammalian and avian genes encoding the fast skeletal muscle isoform, MLC2f. In situ hybridization and Northern blot hybridization analyses indicated that the zebrafish MLC2f mRNA is expressed exclusively in the fast skeletal muscle. Ontogenetically, the MLC2f mRNA appears around 16 hours postfertilization (hpf) in the first few well-formed anterior somites. At later stages, the MLC2f mRNA can also be detected in fin buds, eye muscles, and jaw muscles. To develop a useful model system for analyzing muscle gene regulation, the **promoter of the zebrafish MLC2f gene was isolated and linked to the chloramphenicol acetyltransferase (CAT) reporter gene**. The MLC2f/CAT chimeric constructs were analyzed by direct injection into the zebrafish skeletal muscle, and significant CAT activity was

> observed; in contrast, little or no CAT activity was generated from a similarly injected prolactin gene promoter/CAT gene construct. Within the 1 kb of the MLC2f promoter region, several MEF2-binding sites and E-boxes were identified, suggesting that MLC2f can be regulated by muscle transcription factors MEF2 and myogenic bHLH proteins. A 5' deletion analysis indicated that the proximal 79 nucleotides from the transcription start site, which contains a single MEF2-binding site, is sufficient to drive a high level of CAT activity in injected muscle. Internal deletion of the MEF2 element in the - 79-bp construct caused an 80% decrease in CAT activity, whereas internal deletion of the same MEF2 element in a - 1044-bp construct had no effect on induced CAT activity. These observations suggest that an MEF2 element is important to activate the MLC2f gene in muscle cells, and the effect of loss of the proximal MEF2 element can be compensated for by the presence of the upstream MEF2 elements. This study also demonstrated that direct injection of DNA into skeletal muscle is a valid and valuable approach to analyze muscle gene promoters in the zebrafish. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in glial cells include the following:

1) Zakin MM, Baron B, and Guillou F. (2002). Regulation of the tissue-specific expression of transferrin gene. Dev Neurosci. 24:222-6. Unite d'Expression des Genes Eucaryotes, Institut Pasteur, Paris, France.

> **Transferrin** (Tf), the plasma protein involved in iron transport, seems to play complex physiological roles related to cell function, differentiation and proliferation. The protein is essentially synthesized in hepatocytes, but also in Sertoli cells, in the epithelial cells of the choroid plexus in rodents and in oligodendrocytes in all species analyzed. In this manuscript, we review the results obtained on Tf gene expression in the different cellular systems in which the protein is synthesized. In vitro and ex vivo experiments indicate that different combinations of transcription factors are necessary in different subsets of cells to achieve Tf tissue-specific expression. **Several lines of transgenic mice were generated in which the expression of reporter genes is under the control of different Tf regulatory regions**. More recently, transgenic mice were obtained using the complete human Tf gene and its 5' and 3' flanking sequences. These **mice** constitute the first **model** in which the physiological consequences of a **specific Tf over expression in oligodendrocytes** can be studied. Although much information is now available, further work is still necessary for a full understanding of the in vivo mechanisms responsible for the regulation of Tf gene expression. (Emphasis added)

2) Forghani R, Garofalo L, Foran DR, Farhadi HF, Lepage P, Hudson TJ, Tretjakoff I, Valera P, Peterson A. (2001). A distal upstream enhancer from the myelin basic protein gene regulates expression in myelin-forming schwann cells. J Neurosci. 21:3780-7. Laboratory of Developmental Biology, Department of Neurology and Neurosurgery, Molecular Oncology Group H-5, McGill University, Montreal, Quebec, Canada, H3A 1A1.

> In peripheral nerves, large caliber axons are ensheathed by myelin-elaborating Schwann cells. Multiple lines of evidence demonstrate that expression of the genes encoding myelin structural proteins occurs in Schwann cells in response to axonal instructions. To gain further insight into the mechanisms controlling myelin gene expression, we used reporter constructs **in transgenic mice** to search for the DNA elements that regulate the myelin basic protein (MBP) gene. Through this in vivo investigation, we provide evidence for the participation of multiple, widely distributed, positive and negative elements in the overall control of MBP expression. Notably, all constructs bearing a **0.6 kb far-upstream sequence, designated Schwann cell enhancer 1 (SCE1), expressed at high levels in myelin-forming Schwann cells**. In addition, robust targeting activity conferred by SCE1 was shown to be independent of other MBP 5' flanking sequence. These observations suggest that SCE1 will make available a powerful tool to drive transgene expression in myelinating Schwann cells and that a focused analysis of the SCE1 sequence will lead to the identification of transcription factor binding sites that positively regulate MBP expression. (Emphasis added)

3) Belachew S, Yuan X, Gallo V. (2001). Unraveling oligodendrocyte origin and function by cell-specific transgenesis. Dev Neurosci. 23:287-98. Laboratory of Cellular and Synaptic Neurophysiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4495, USA. sbelachew@ulg.ac.be

> Besides the role of mature oligodendrocytes in myelin synthesis during the development of the central nervous system (CNS), the oligodendrocyte lineage also encompasses the largest pool of postnatal proliferating progenitors whose behavior in vivo remains broadly elusive in health and disease. We describe here transgenic models that allow us to track the functions and origins of such cells by using **proteolipid protein** and 2',3'-cyclic nucleotide 3'-phosphodiesterase (**CNP**) gene **promoters** to **direct oligodendroglial expression** of different reporters, in particular the green fluorescent protein (GFP). We emphasize that the **CNP-GFP mouse**, which **targets the entire oligodendroglial lineage** from embryonic life to adulthood, provides an outstanding tool to study the in vivo properties of oligodendrocyte progenitor cells in normal and damaged CNS. (Emphasis added)

4) Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. J Neurosci 14:1030-1037. Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892.

> Glial fibrillary acidic protein (**GFAP**) is an intermediate-filament protein expressed abundantly and almost **exclusively in astrocytes** of the CNS. We are studying transcriptional regulation of the GFAP gene to gain insight into astrocyte function and also to develop an astrocyte- specific expression system for manipulating brain physiology. In this work, we have produced **transgenic mice** carrying the bacterial lacZ reporter gene linked to a **2.2 kilobase 5'-flanking sequence derived from the human GFAP gene** that previously was **shown to direct astrocyte- specific transcription** in cultured cells. We report that this promoter directs expression to astrocytes in the CNS. In addition, the upregulation of GFAP gene activity that follows injury to the brain was mimicked by the transgene. One of the transgenes was found to be X- linked and appeared to undergo the usual random inactivation that achieves gene dosage compensation in females. The brains of hemizygous females stained uniformly rather than displaying mosaic patches, indicating that astrocytes intermingle following their formation. The specific expression of the GFAP-lacZ transgene means that it is now possible to target expression of other heterologous genes to astrocytes in vivo, and to study the mechanisms for reactive gliosis at the DNA level. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in pancreatic cells include the following:

1) Herrera PL. (2002). Defining the cell lineages of the islets of Langerhans using transgenic mice. Int J Dev Biol. 46:97-103. Department of Morphology, University of Geneva Medical School, Switzerland.

> In this Special Issue of the Int. J. Dev. Biol., we summarize our own studies on the development of the **mouse endocrine pancreas**, with special emphasis on the cell lineage relationships between the **four islet cell types**. Considerable knowledge concerning the ontogeny of the endocrine pancreas has been gained in recent years, mainly through the use of two complementary genetic approaches in mice: gene inactivation and genetic labelling of precursor cells. However, neither gene inactivation in KO mice nor co-localisation of hormones in single cells during development can be taken as evidence for cell lineage relationships among different cell types. The **beta-cell lineage** analysis was started by selectively ablating specific

> islet cell types in **transgenic mice**. We used the diphtheria toxin A subunit coding region under the control of **insulin, glucagon or pancreatic polypeptide (PP) promoters**, in order to eliminate **insulin-, glucagon- or PP-expressing cells, respectively**. Contrary to the common view, we demonstrated that glucagon cells are not precursors of insulin-producing cells. These results were in addition the first evidence of a close ontogenetic relationship between insulin and somatostatin cells. We pursued these analyses using a novel, more subtle approach: progenitor cell labelling through the expression of Cre recombinase in doubly transgenic mice. We were able to unequivocally establish that 1) adult glucagon- and insulin-producing cells derive from precursors which have never transcribed insulin or glucagon, respectively; 2) insulin cell progenitors, but not glucagon cell progenitors transcribe the PP gene and 3) adult glucagon cells derive from progenitors which do express pdx1. (Emphasis added)

2) Herrera PL, Orci L, and Vassalli JD. (1998). Two transgenic approaches to define the cell lineages in endocrine pancreas development. Mol Cell Endocrinol. 140:45-50. Department of Morphology, Faculty of Medicine, Geneva, Switzerland.

> Ontogenic relationships between the different endocrine cell types of the islets of Langerhans were explored by generating **transgenic mice**, in which **cells transcribing the glucagon, insulin, or pancreatic polypeptide genes** were destroyed through the **promoter-targeted** expression of the diphtheria toxin A chain. In an alternate approach, to assess whether insulin cells are derived from precursors producing glucagon or PP, transgenic mice were generated bearing an insulin promoter-driven, and loxP-containing ('floxed') reporter transgene that can be irreversibly 'tagged' by recombination. They were crossed with mice expressing another transgene ('tagger') encoding Cre (cyclization recombination) recombinase in either glucagon or PP cells. The results obtained using both approaches indicate that neither glucagon nor insulin gene-expressing cells are the precursors to the other islet cells; also, they suggest that PP gene-expressing cells are necessary for the differentiation of islet insulin and somatostatin cells, through a cell lineage or a paracrine relationship. (Emphasis added)

3) Gannon M, Gamer LW, and Wright CV. (2001). Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene pdx1. Dev Biol. 238:185-201. Department of Cell Biology, Vanderbilt University Medical Center, 1161 21st Avenue South, Nashville, Tennessee 37232, USA.

> **pdx1** (pancreatic and duodenal homeobox gene-1), which is **expressed broadly in the embryonic pancreas and, later, in**

> **a more restricted manner in the mature beta cells in the islets of Langerhans**, is essential both for organ formation and beta cell gene expression and function. We carried out a transgenic reporter gene analysis to **identify region- and cell type-specific regulatory regions in pdx1**. A 14.5-kb pdx1 genomic fragment corrected the glucose intolerance of pdx1 (+/-) animals but, moreover, fully rescued the severe gut and pancreas defects in pdx1(-/-) embryos. **Sequences sufficient to direct reporter expression to the entire endogenous pdx1 expression domain lie within 4.3 kb of 5' flanking DNA**. In this region, we identified two distinct fragments that drive reporter gene expression to different sets of islet neuroendocrine cells. One shows pan-endocrine cell specificity, the other is selectively activated in insulin-producing beta cells. The endocrine-specific regulatory regions overlap a localized region of 5' flanking DNA that is remarkably conserved in sequence between vertebrate pdx1 genes, and which has been associated with beta cell-selective expression in cultured cell lines. This region contains potential binding sites for several transcription factors implicated in endodermal development and the pathogenesis of some forms of type-2 diabetes. These results are consistent with our previous proposal that conserved upstream pdx1 sequences exert control over pdx1 during embryonic organogenesis and islet endocrine cell differentiation. We propose that mutations affecting the expression and/or activity of transcription factors operating via these sequences may predispose towards diabetes, at least in part by direct effects on endocrine pdx1 expression. (Emphasis added)

4) Beck CW & Slack JM (1999). Gut specific expression using mammalian promoters in transgenic Xenopus laevis. Mech Dev. 88:221-7. Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath, UK. c.beck@bath.ac.uk

> The recent development of **transgenic** methods for the **frog** Xenopus laevis provides the opportunity to study later developmental events, such as organogenesis, at the molecular level. Our studies have focused on the development of the tadpole gut, where tissue specific promoters have yet to be identified. We have **used mammalian promoters, for the genes elastase, pancreatic duodenal homeobox-1, transthyretin, and intestinal fatty acid binding protein to drive green fluorescent protein expression in live tadpoles. All of these were shown to drive appropriate tissue specific expression**, suggesting that the molecular mechanisms

> organising the gut are similar in amphibians and mammals. Furthermore, expression from the **elastase promoter** is initiated in the pancreatic buds before morphological definition becomes possible, making it a powerful **tool for the study of pancreatic determination**. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in liver cells include the following:

1) J. Wijnholds, S Philipsen, S Pruzina, P Fraser, F Grosveld, and G Ab (1993). Estrogen-inducible and liver-specific expression of the chicken Very Low Density Apolipoprotein II gene locus in transgenic mice. Nucleic Acids Res. 1993 April 11; 21(7): 1629–1635.

> We have examined the chicken Very Low Density Apolipoprotein II (apoVLDL II) gene locus in transgenic mice. A DNA fragment composed of the transcribed region, 16 kb of 5' flanking and 400 bp of 3' flanking sequences contained all the information sufficient for estrogen-inducible, liver-specific expression of the apoVLDL II gene. The far-upstream region contains a Negative Regulating Element coinciding with a DNaseI-hypersensitive site at -11 kb. In transgenic mice, the NRE at -11 kb is used for downregulating the expression to a lower maximum level. The NRE might be used for modulating apoVLDL II gene expression, and may be involved in the rapid shut-down of the expression after hormone removal.

2) Pinkert CA, Ornitz DM, Brinster RL, Palmiter RD. (1987). An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. J Genes Dev. May;1(3):268-76. Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, 19104.

> Transgenic mice were used to locate the cis-acting DNA elements that are important for efficient, tissue-specific expression of the mouse albumin gene in the adult. Chimeric genes with up to 12 kb of mouse albumin 5'-flanking region fused to a human growth hormone (hGH) reporter gene were tested. Remarkably, a region located 8.5-10.4 kb upstream of the albumin promoter was essential for high-level expression in adult liver and the region in between -8.5 and -0.3 kb was dispensable. The far-upstream region behaved like an enhancer in that its position and orientation relative to the albumin promoter were not critical; however, it did not function well with a heterologous promoter. Two of four DNase hypersensitive sites found in the 5'-flanking region of the albumin gene map to the far-upstream and promoter regions;

the others may reflect regions involved in developmental or environmental control of this gene.

3) Idzerda RL, Behringer RR, Theisen M, Huggenvik JI, McKnight GS, Brinster RL. (1989). Expression from the transferrin gene promoter in transgenic mice. Mol Cell Biol. 1989 Nov;9(11):5154-62. Department of Pharmacology, School of Medicine, University of Washington, Seattle 98195.

Transferrin is an iron-binding protein that is expressed as a major product in liver and secreted into the plasma. To study the tissue-specific regulatory regions of this gene, the genomic mouse transferrin (mTf) gene was cloned and characterized by partial sequence analysis and S1 nuclease mapping of the transcriptional start site. Fusion genes containing the transferrin gene promoter and 5'-flanking sequences were ligated to the human growth hormone (hGH) gene and used to produce transgenic mice. A deletion construct containing the -581 to +50 region of the transferrin gene was sufficient to direct a high level of liver-specific expression resembling endogenous transferrin gene expression. Deletion to -139 base pairs of 5'-flanking sequence gave a construct which retained liver specificity, but the magnitude of expression decreased severalfold. These results demonstrate the presence of a liver-specific transcriptional element between -139 and +50 and suggest the presence of a distal element between -581 and -139 that can further increase expression. Surprisingly, fusion constructs containing -3 kilobase pairs (kb) of 5'-flanking sequence gave higher levels of mRNA in nonhepatic tissues than did either the -581 or -139 construct. Further studies indicated that the high levels of circulating hGH in these transgenic mice specifically induced the endogenous transferrin and albumin genes in liver and also stimulated the normally low levels of expression of the endogenous transferrin gene in brain, heart, kidney, and muscle. A mutated hGH gene that does not produce active growth hormone was fused to the -3- to +50-kb transferrin sequences to produce the -3-kb mTf-hGX construct. A liver-specific pattern of expression was observed in transgenic mice harboring the -3-kb mTf-hGX construct, and this mutated transgene was shown to be induced four- to sevenfold by either bovine or human growth hormone. These results demonstrate the presence of a growth hormone-responsive element between -3 and +50 kb in the 5'-flanking region of the mTf gene promoter.

4) Shachter, N. S., Y. Zhu, A. Walsh, J. L. Breslow, and J. D. Smith. (1993). Localization of a liver-specific enhancer in the apolipoprotein E/C-I/C-II gene locus. J. Lipid Res. 34: 1699-1707.

> The sequences necessary for liver-specific expression of the apolipoprotein (apo) E gene have been shown to reside 3' to the gene, within the apoE/C-I/C-I1 gene cluster, but have not been precisely characterized. Utilizing a transient transfection reporter gene assay based on the apoC-I1 promoter, we have localized a liver-specific enhancer to its approximate limit dimension of 154 base pairs. This enhancer directed liverspecific expression of an apoE gene construction in transgenic mice. A DNase1 protection assay revealed two footprints over an inverted repeat of a known transcriptionally active motif, TGACCT. DNaseI-sensitive sites were present in three of six repeats of a motif (consensus GCAAACA) which has been postulated to represent the recognition sequence of a hepatic transcriptional activity, HNF-5. This region of DNA may function as a liver-specific enhancer for the entire apoE/C-I/C-I1 gene cluster.

References for regulatory sequences driving transgene expression specifically in kidney cells include the following:

1) Igarashi P, Shashikant CS, Thomson RB, Whyte DA, Liu-Chen S, Ruddle FH, Aronson PS. (1999). Ksp-cadherin gene promoter. II. Kidney-specific activity in transgenic mice. Am J Physiol. 1999 Oct;277(4 Pt 2):F599-610. Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA. igarashi@email.swmed.edu

> Kidney-specific cadherin (Ksp-cadherin, cadherin 16) is a tissue-specific member of the cadherin superfamily that is expressed exclusively in the basolateral membrane of tubular epithelial cells in the kidney. To determine the basis for tissue-specific expression of Ksp-cadherin in vivo, we evaluated the activity of the promoter in transgenic mice. Transgenic mice containing 3.3 kb of the mouse Ksp-cadherin promoter and an Escherichia coli lacZ reporter gene were generated by pronuclear microinjection. Assays of beta-galactosidase enzyme activity showed that the transgene was expressed exclusively in the kidney in both adult and developing mice. Within the kidney, the transgene was expressed in a subset of renal tubular epithelial cells that endogenously expressed Ksp-cadherin and that were identified as collecting ducts by colabeling with Dolichos biflorus agglutinin. In the developing metanephros, expression of the transgene in the branching ureteric bud correlated with the developmental expression of Ksp-cadherin. Identical patterns of expression were observed in multiple founder mice, indicating that kidney specificity was independent of transgene integration site. However, heterocellular expression was observed consistent with repeat-induced gene silencing. We conclude that the Ksp-cadherin

> gene promoter directs kidney-specific expression in vivo. Regulatory elements that are sufficient to recapitulate the tissue- and differentiation-specific expression of Ksp-cadherin in the renal collecting duct are located within 3.3 kb upstream to the transcriptional start site.

2) Zhu X, Cheng J, Gao J, Lepor H, Zhang ZT, Pak J, Wu XR. (2002). Isolation of mouse THP gene promoter and demonstration of its kidney-specific activity in transgenic mice. Am J Physiol Renal Physiol. 2002 Apr;282(4):F608-17. Department of Urology, Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York 10016, USA.

> Tamm-Horsfall protein (THP), the most abundant urinary protein synthesized by the kidney epithelial cells, is believed to play important and diverse roles in the urinary system, including renal water balance, immunosuppression, urinary stone formation, and inhibition of bacterial adhesion. In the present study, we describe the isolation of a 9.3-kb, 5'-region of the mouse THP gene and show the highly conserved nature of its proximal 589-bp, 5'-flanking sequence with that in rats, cattle, and humans. We also demonstrate using the transgenic mouse approach that a 3.0-kb, proximal 5'-flanking sequence is sufficient to drive the kidney-specific expression of a heterologous reporter gene. Within the kidney, transgene expression was confined to the renal tubules that endogenously expressed the THP protein, which suggests specific transgene activity in the thick ascending limb of the loop of Henle and early distal convoluted tubules. Our results establish the kidney- and nephron-segment-specific expression of the mouse THP gene. The availability of the mouse THP gene promoter that functions in vivo should facilitate additional studies of the molecular mechanisms of kidney-specific gene regulation and should provide new molecular tools for better understanding renal physiology and disease through nephron-specific gene targeting.

3) Pfeffer PL, Payer B, Reim G, di Magliano MP, Busslinger M. (2002). The activation and maintenance of Pax2 expression at the mid-hindbrain boundary is controlled by separate enhancers. Development. 2002 Jan;129(2):307-18. Research Institute of Molecular Pathology, Vienna Biocenter, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria.

> Pax2 is the earliest known gene to be expressed throughout the mid-hindbrain region in late gastrula embryos of the mouse and is essential for the formation of an organizing center at the midbrain-hindbrain boundary (MHB), which controls midbrain and cerebellum development. We have used transgenic analysis to identify three MHB-specific enhancers in the upstream region of the mouse Pax2 gene. A 120 bp enhancer (at -3.7 kb)

> in cooperation with the endogenous promoter was sufficient to induce transgene expression in the anterior neural plate of late gastrula embryos, while it was already inactivated again at the MHB during somitogenesis. The activity of this early enhancer was severely reduced by mutation of three homeodomain-binding sites, two of which are part of a recognition sequence for POU homeodomain proteins. Oct3/4 (Pou5f1), the mouse ortholog of zebrafish Pou2, efficiently bound to this sequence, suggesting its involvement in the regulation of the early Pax2 enhancer. Starting at the four-somite stage, Pax2 is expressed at the MHB under the control of two enhancers located at -4.1 kb and -2.8 kb. The distal late enhancer contains a 102 bp sequence that is not only highly conserved between the mouse and pufferfish Pax2 genes, but also contributes to the enhancer activity of both genes in transgenic mice. The proximal 410 bp enhancer, which overlaps with a **kidney-specific regulatory element**, contains a functional Pax2/5/8-binding site and thus maintains Pax2 expression at the MHB under auto- and cross-regulatory control by Pax2/5/8 proteins. Importantly, the early and proximal late enhancers are not only sufficient but also necessary for expression at the MHB in the genomic context of the Pax2 locus, as their specific deletion interfered with correct temporal expression of a large Pax2 BAC transgene. Hence, separate enhancers under the control of distinct transcription factors activate and maintain Pax2 expression at the MHB. (Emphasis added)

References for regulatory sequences driving transgene expression specifically in vascular cells include the following:

1) Lilly B, Olson EN, Beckerle MC. (2001). Identification of a CArG box-dependent enhancer within the cysteine-rich protein 1 gene that directs expression in arterial but not venous or visceral smooth muscle cells. Dev Biol. 240:531-47. The Huntsman Cancer Institute and Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA. brenda.lilly@hci.utah.edu

> Smooth muscle cells (SMCs) are heterogeneous with respect to their contractile, synthetic, and proliferative properties, though the regulatory factors responsible for their phenotypic diversity remain largely unknown. To further our understanding of smooth muscle gene regulation, we characterized the cis-regulatory elements of the murine cysteine-rich protein 1 gene (CRP1/Csrp1). CRP1 is expressed in all muscle cell types during embryogenesis and predominates in vascular and visceral SMCs in the adult. We identified a 5-kb enhancer within the CRP1 gene that is sufficient to drive expression in arterial but not venous or visceral SMCs in transgenic mice. This enhancer also exhibits region-specific activity in the

> outflow tract of the heart and the somites. Within the 5-kb CRP1 enhancer, we found a single CArG box that binds serum response factor (SRF), and by mutational analysis, demonstrate that the activity of the enhancer is dependent on this CArG element. Our findings provide further evidence for the existence of distinct regulatory programs within SMCs and suggest a role for SRF in the activation of the CRP1 gene.

2) Moessler H, Mericskay M, Li Z, Nagl S, Paulin D, Small JV. (1996). The SM 22 promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. Development.122:2415-25. Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria.

> The transcriptional signals underlying smooth muscle differentiation are currently unknown. We report here the complete sequence and characterization of the single mouse gene for the smooth muscle-specific protein SM 22 and the transcriptional activity of its promoter in cultured smooth muscle cells in vitro and in transgenic mice. In the transgenic animals, promoter constructs ranging in length from 445 to 2126 bp directed reporter expression initially in the heart and the somites of embryos and subsequently in the arteries of the vascular system, but in none of the visceral muscles, nor in the veins. Expression in the heart was spatially restricted to the presumptive right ventricle and outflow tract and disappeared in the adult. Likewise, expression in the somites was only transitory and was not observed after about 14.5 days post coitum in the embryo. In the adult mouse, SM 22 promoter activity persisted in the smooth muscle cells of the arteries and was still notably absent from other smooth muscles, despite the ubiquitous presence of the endogenous SM 22 protein. These findings on the transcriptional activity of a smooth muscle promoter in vivo reveal the existence of different differentiation programmes for smooth muscle cells in the veins and the arteries and raise the expectation of a further subdivision of programmes among the visceral muscles.

3) Keys JR, Greene EA, Koch WJ, Eckhart AD. (2002) Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. Hypertension. 40(5):660-6. Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA.

> The Gq-coupled receptor-signaling pathway has been implicated in the cardiac hypertrophic response to stress, but little is actually known about the contributions of Gq signaling in either the heart or the vasculature. Therefore, we developed a **line of transgenic mice that express a peptide inhibitor of Gq (GqI) in vascular smooth muscle** to determine if vascular Gq signaling was important in the cardiac hypertrophic response. After chronic administration of the Gq agonists

> phenylephrine, serotonin, and angiotensin II, we observed an attenuation of mean arterial blood pressure and an inhibition of cardiac hypertrophy in the transgenic mice with **vascular-specific GqI expression**. In contrast, cardiac GqI peptide expression did not attenuate the hypertension or the cardiac hypertrophy. Importantly, all mice were capable of cardiac hypertrophy, because direct beta-adrenergic receptor stimulation induced a similar level of hypertrophy in both lines of transgenic mice. This clearly suggests that after chronic Gq-coupled receptor agonist administration, it is the hypertensive state induced by vascular Gq activation that mediates remodeling of the heart, rather than direct stimulation of cardiac Gq-coupled receptors. Thus, the contribution of vascular Gq-coupled signaling to the development of cardiac hypertrophy is significant and suggests that expression of the GqI peptide is a novel therapeutic strategy to lower Gq-mediated hypertension and cardiac hypertrophy. (Emphasis added)

4) Korhonen J, Lahtinen I, Halmekytö M, Alhonen L, Jänne J, Dumont D, Alitalo K. (1995). Endothelial-specific gene expression directed by the tie gene promoter in vivo. Blood. 86(5):1828-35. Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, Finland.

> The tie gene encodes a receptor tyrosine kinase that is expressed in the endothelium of blood vessels, particularly during embryonic development and angiogenesis in adults. We have cloned and characterized the mouse tie gene and isolated the human and mouse tie promoters. The **promoter activities of human and mouse tie were analyzed using luciferase reporter gene** constructs in transfected cell lines and beta-galactosidase constructs in **transgenic mice**. In transfection assays of cultured cells, both human and mouse promoter DNA fragments showed activity that was not restricted to endothelial cells. In contrast, **in transgenic mice both promoters directed expression of the reporter gene to endothelial cells undergoing vasculogenesis and angiogenesis.** In adult mice, tie promoter activity in lung and many vessels of the kidney was as high as in the vessels of the corresponding embryonic tissues, whereas in the heart, brain and liver, tie promoter activity was downregulated and restricted to coronaries, cusps, capillaries, and arteries. Our results show that the endothelial cell-type specificity of the tie promoter in vivo can be transferred to heterologous genes by using relatively short promoter fragments. The tie promoter, thus, has useful properties for potential gene therapy. (Emphasis added)

Accordingly, Applicant submits that former Claims 38-40 and newly added Claims 43-50 are supported by the specification and comply with the written description requirement.

For at least the reasons set forth above, Applicant respectfully requests that the Section 112 rejection of Claims 38-40 be withdrawn.

The rejection of Claims 31-42 under 35 U.S.C. § 112, first paragraph, as not being enabled is respectfully traversed. Claims 32, 35, and 37-42 have been canceled. Applicant has amended Claim 31 to include the limitation "a regulatory DNA sequence, including at least a promoter element that regulates the expression of the ablation promoting moiety". Accordingly, Applicant has addressed the issue raised in the Office Action and, therefore, submits that Claim 31 is enabled.

Claims 32, 35, and 37-42 have been canceled. Claims 33, 34, and 36 depend from independent Claim 31. When the recitations of Claims 33, 34, and 36 are considered in combination with the recitations of Claim 31, Applicant submits that Claims 33, 34, and 36 likewise are enabled.

For at least the reasons set forth above, Applicant respectfully requests that the Section 112 rejection of Claims 31-42 be withdrawn.

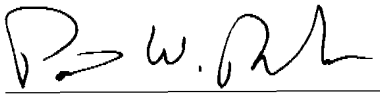
The rejection of Claims 35, 37, and 41-42 under 35 U.S.C. § 112, second paragraph, as being indefinite is respectfully traversed. Claims 35, 37, 41 and 42 have been canceled. Accordingly, Applicant respectfully requests that the Section 112 rejection of Claims 35, 37, and 41-42 be withdrawn.

With respect to newly added Claims 43-51, Applicant submits that Claims 43-51 are patentable for the reasons set forth above. Specifically, Claims 43 and 44 are dependent on Claim 31 and, therefore, are submitted to be patentable for the reasons that correspond to the patentability of Claim 31. Further, Claims 45, 48, and 51 include recitations that are essentially similar to the recitations of Claim 31 and, therefore, are also submitted to be patentable for the reasons that correspond to the patentability of Claim 31. Moreover, Claims

46 and 47 are dependent from Claim 45, Claims 49 and 50 are dependent from Claim 48. Accordingly, Claims 46, 47, 49, and 50 are submitted to be patentable for the reasons that correspond to the patentability of Claims 45 and 48, respectively.

In view of the foregoing amendment and remarks, all the claims now active in this application are believed to be in condition for allowance. Reconsideration and favorable action is respectfully solicited.

Respectfully Submitted,



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